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Effect of ionizing radiation induced damage and protection in normal and denatured supercoiled DNA and minichromatins formed on them

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Abstract · Minichromatins can be generated on supercoiled Form I plasmid DNA as well as on a compact denatured supercoiled structure (Form I_d). On using ionizing radiation, ^{60}Co-γ-rays and ^{16}O-heavy ion beam, the Form I_d shows a strong resistance to damage as compared to Form I DNA. However, when hydroxyl radical produced through Fenton's reaction is used for the study, there is an extensive damage to the Form I_d structure. Similar studies with minichromatins show strong resistance to all the probes used.

Keywords Denatured supercoiled DNA ; minichromatin ; Heavy ion ; γ-ray ;

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1. Introduction

The compaction of DNA in eukaryotes and to some extent in prokaryotic system, has often been connected to the ability of the DNA being protected from various physical agents. The current studies involve the breakage produced by heavy ions, γ-irradiation using the substrates namely supercoiled plasmid DNA, alkali denatured supercoiled DNA (Form I_d), a form with a secondary structure different from Form I and the minichromatins generated on them. The main aim of this study is to assess the effect of those physical agents on altered and compact structure of plasmid DNA *in vitro*.

Form I_d is a condensed structure following collapse of the DNA structure after treatment with alkali [1]. It has a characteristic buoyant density [2], lower specific viscosity [3] and CD spectrum [4]. The compactness of the structure is maintained even on neutralization. Form I_d moves at a much faster rate than the Form I plasmid DNA. It has been shown to be the better substrate than Form I template with regard to semi *in vitro* replication [5]. We were interested in generating minichromatins on Form I and Form I_d to get a structure partially mimicking the *in vivo* situation. This structure has been tested for the sensitivity to radiation induced damage

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and the results will be compared to damage inflicted on chromatin. Further, plasmids bearing these structures have been subjected to damage causing events in order to assess the role of structure modification or proteins on the extent of damage produced. Any repair process initiated following radiation damage by ionizing radiation involves recombinogenic repair pathway when replication process is expected to be activated.

Regarding the nature of the heavy ion, the velocity of the projectile exceeds the orbital velocity of its own electrons, the latter electrons are stripped off in the first few collisions and only the stronger bound electrons of the inner shell remain. As the heavy ions start slowing down, more and more electrons are captured until the charged state is neutralised. The inner part of the structure of the track is formed mainly by the low energy electrons and the outer part by the high energy electrons [6]. Heavy ions exhibit a completely different dose profile in tissue with respect to penetration depth when compared to corresponding electromagnetic radiations used in standard radiotherapy. In the aqueous environment of tissues reactive radicals are produced from the molecules adjacent to the centre of the impingement. Heavy charged particles have two advantages. The first is : particles have the finite range of impingement and increased dose deposition towards the end of the particle track, the second: heavy ions exhibit an increased biological efficiencies in the vicinity of high energy deposition [7].

For the γ-rays, neutrons etc., the dose decreases exponentially after penetrating a few centimeters whereas for the heavy charged particles the dose or energy of deposition attains a maximum after it penetrates a depth of the target, known as Bragg Peak, which is usually just before the end of the primary particle range. The ionizing radiation is known to damage DNA through three methods 1) direct, 2) quasi-direct and 3) in -direct effect. In direct effect the DNA itself is damaged ; in quasi-direct, ionization of tightly bound water molecule leads to damage of the DNA ; in the indirect effect radiolysis of the solution surrounding the DNA gives rise to reactive radical species which subsequently react with the DNA. The role of OH. radical scavengers in inhibiting the damaging effect shows that these are the principal species for indirect effect [8]. The damage to the DNA is complex in the sense that majority of the products are damaged bases ; other products include single-strand breaks (SSBs) and double strand breaks (DSBs).

Among the lesions induced by ionizing radiation in cellular DNA, double-strand breaks (DSBs) are the least efficiently repaired, and their frequency is often correlated with cell death [9]. DSBs are noninformative lesions that affect the DNA double helix at the same site, eliminating intact template for repair and precluding any excision repair. In D. radiodurans, interchromosomal recombination has been suggested as the major repair pathway for chromosomal mending alongwith a singlestrand annealing repair mechanism immediately following irradiation. In this organism, the onset of recombination and growth are separated by about 7h, suggesting that extensive recombination repair occurs before DNA replication [10]. However the recombination process often involves a host of proteins that control replication as well.

Replication is an important event especially in highly proliferating tissues, both in cancerous growth as well as in developmental stage. At the replication fork numerous proteins constitute preprimosomal structure. It has been previously suggested that the histone proteins partially protect the DNA structure from being damaged by various agents as for example from

Plate I

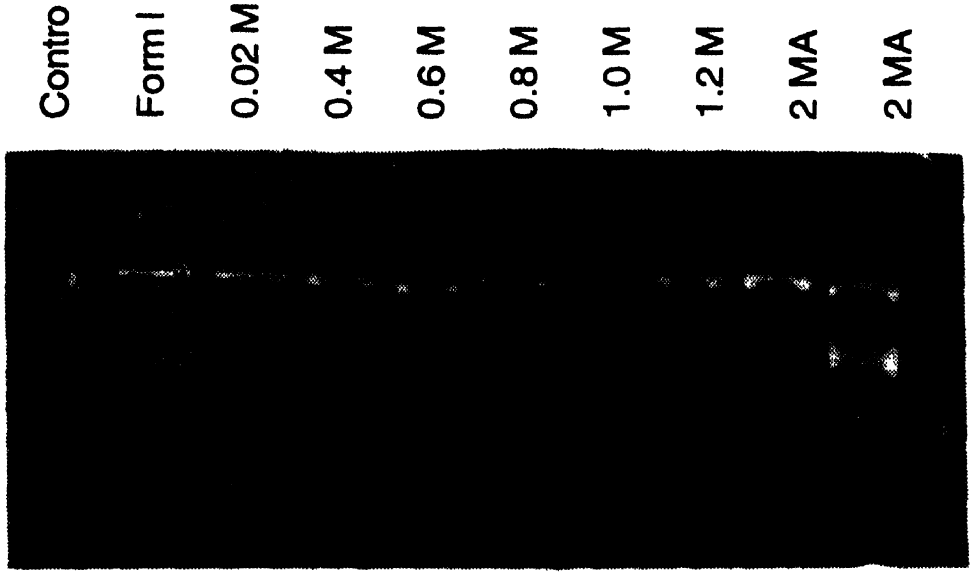

Figure 1. Reconstitution of minichromatin on Form I supercoiled DNA , the salt concentration are indicated on the panels above the lanes

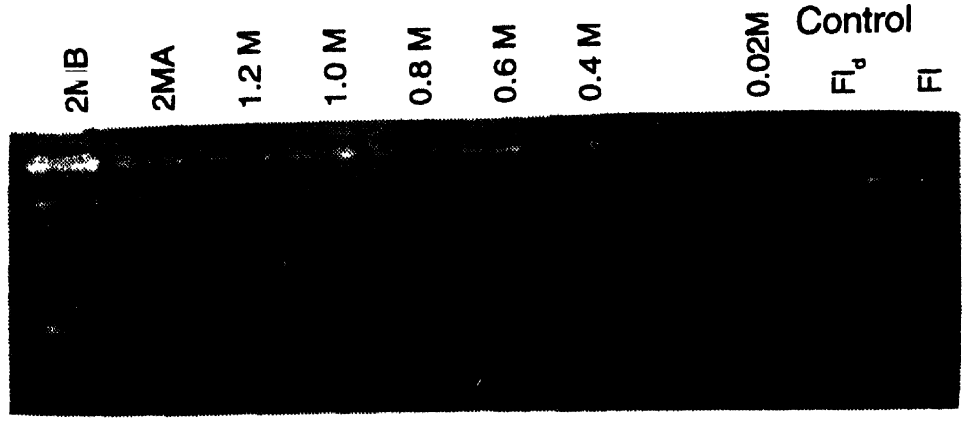

Figure 2. Reconstitution of minichromatin on Form I_d DNA at different salt concentration The faster migrating form than Form I DNA is the Form I_d.

the attack of the hydroxyl radical [11, 12]. Actually there are histone like proteins that participate in initiation of replication. Some other proteins generate a novel structure in the case of plasmid Form I, also known as Form I*[13]. It has already been reported that condensed DNA structure in chromatin is considerably less sensitive to radiation damage than the normal DNA or the expanded chromatin characteristic of replicating or transcribing domains [14].

2. Materials and methods

Materials :

Chemicals used for this study were essentially of analytical grade. Trizma base, Ethylenediamine tetraacetate (EDTA), β-Mercaptoethanol (β-ME), low EEO Agarose, Magnesium Chloride, Calcium Chloride, Calf Thymus Nucleohistone, Micrococcal Nuclease were from M/S Sigma Chemicals, St. Louis, USA. Sodium Chloride and Ethanol was from M/s. E Merck (I) Ltd., Bombay, India. Sodium Hydroxide, Sucrose were from Qualigens Fine Chemicals, Bombay, India.

Preparation of denatured supercoiled DNA :

Form I_d was prepared using plasmid pbluescript (2961bp) following the protocol of Santra *et al* [5]. The quality of the plasmid from which Form I_d was produced, was analysed by UV spectra in a Hitachi U2000 spectrophotometer and also by agarose gel electrophoresis. The plasmids contained > 90% supercoiled DNA in most of the experiments. To prepare Form I_d, same volume of 1 M NaOH was added to solution of supercoiled DNA and kept at subzero temperature for 15 minutes. After that twice the volume of 1 M Tris buffer (pH= 8.0) was added to DNA. The preparation was subjected to ethanol precipitation and resuspended in TE buffer (pH = 8.0).

Generation of Minichromatins :

Minichromatins were generated using salt gradient dialysis [16]. The calf thymus nucleohistones were partially digested by Micrococcal Nuclease to monosomes which were subsequently purified on 5-20% sucrose gradients in Tris (10 mM), EDTA (1mM), Mercaptoethanol (1mM) (TEM) buffer. The purified monosomes were either mixed with Form I or Form I_d DNA in 1.0 : 0.8 mass ratio in 2 M NaCl in TEM buffer. These mixtures were dialysed overnight at 4°C against TEM with 2 M NaCl. The next morning the salt concentration was reduced to 1.2 M and the dialysis was continued for 80 minutes. NaCl concentration of the dialysis buffer were reduced gradually to 1.0 M, 0.8 M, 0.6 M and 0.4 M and dialysis was continued for 80 minutes at each concentration of salt at 4°C. The minichromatins were analysed by running the samples on agarose gel (Figures 1 and 2). Analysis of the minichromatins on polyacrylamide gel was not carried out since the plasmid cannot be resolved due to its size.

Preparation of the target :

a) For Heavy Ion Irradiation

Irradiation by charged particle was carried out at the Nuclear Science Centre, using the radiation biology beam line facility utilising the 15 MV pelletron. ^{16}O (68 MeV) was used to bombard samples at the atmospheric pressure. The fluence and energy were measured at the sample site using silicon surface barrier detectors. Online measurement of the fluence was done by

calibrating a monitor detector placed inside a chamber upstream of the beamline. The precise delivery of the dose was done using a computer interfaced to the accelerator control.

The samples were taken on sterile transparency sheets and covered with sterile polypropylene sheets. About 300ng of DNA or minichromatins were used for irradiation. The DNA samples which were exposed for higher doses had long exposure hours which evaporated the samples. To avoid the problem samples were mixed with equal volume of sterile 100% glycerol.

Heavy Ion Irradiation : Calculation of the dose in Gray.

Range of 68 MeV ^{16}O in glycerol = 72 μm, entrance LET = 654 keV/μm approximately. The average absorbed dose in an absorber of thickness d (cm) which is enough to stop the particle and the density ρ (gm/cc) is given by :

$D = 1.602 \times 10^{-10} \times$ fluence (p/cm^2) × E (MeV) / (ρ.d) Gray, where d can be calculated fro the target volume / target area : this is of approximate range value [17] ρ = 1.13 g/ml, d = 6.2×10^{-3} cm.

Table : The course of irradiation

Approx Fluence	Dose in Gray	Form I	Form I_d	Form I MC	Form I_d MC
5×10^6	8	Y	Y	–	
1×10^7	16	Y	Y	–	–
2×10^7	32	Y	Y	–	–
5×10^7	80	Y	Y	Y	Y
1×10^8	160	Y	Y	Y	Y
2×10^8	320	Y	Y	Y	Y
5×10^8	800	Y	Y	Y	Y

Y = irradiated, – = not irradiated

b) For γ-irradiation

The samples each containing 500 ng of DNA were taken in 500 μl microfuge tube and irradiated in ice for different doses. The dose rate of ^{60}Co-γ-rays were 4 Gray / minute. The nature of damage was standardised and 30, 60 and 120 Gray were chosen.

Analysis of the damage

The damages were analysed running the samples on 1% agarose gel containing ethidium bromide at 50 V for 3 hrs. Almost entire sample that was recovered from the polypropylene sheet was loaded on the grooves. The gels on completion of electrophoresis were photographed and scanned using densitometer.

3. Results and discussions

The nature of minichromatins :

From the result cited at Figure 1 it is clear that almost entire duplex Form I has generated a retarded species which we specified as minichromatin. The histone octamers wrapped perhaps

Plate II

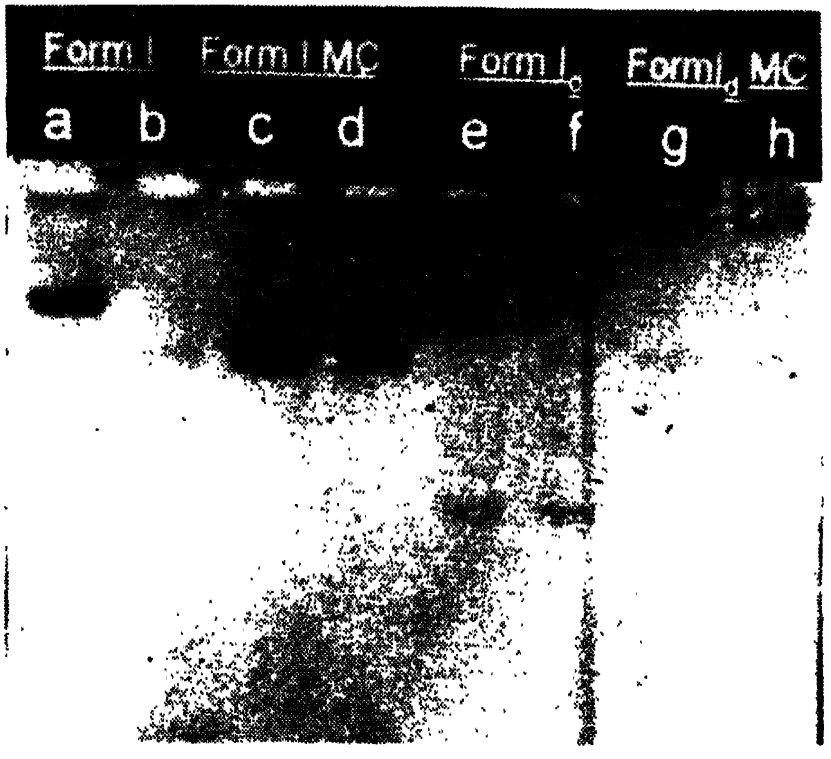

Figure 3. ^{16}O -Beam induced breakage of supercoiled Form I and protection of Form I_d and minichromatins of Form I and Form I_d. The lanes a, c, e, g indicate control samples. The lanes b, d, f, h indicate samples irradiated at dose = 5×10^8 p/cm^2

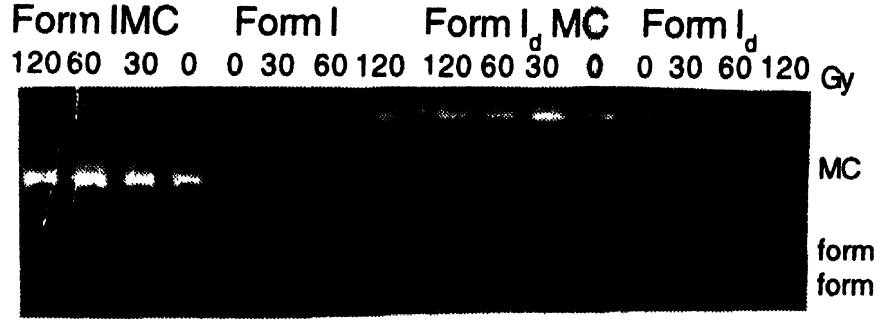

Figure 4. γ-ray induced damage of Form I and protection of Form I_d and minichromatins on Form I and Form I_d. The doses in Gray and the sample type are indicated on the panels above the lanes.

the entire perimeter of the supercoiled DNA. From Figure 2 it is observed that for the Form I_d the minichromatins were not so well formed as it happened in case of duplex DNA. The Form I_d DNA has wide single stranded region which may not be favourable substrate for histone to wrap around.

The heavy ion irradiation :

Figure 3 shows the damage of DNA caused by increasing fluence of the ^{16}O beam. The densitometric scan of the negative of the above photograph is also cited (Figure 5). At lower doses (~ 5×10^6 P/cm^2) the single strand nick was observed while at higher doses the double strand break became prominent (at fluences 5×10^7, 1×10^8, 2×10^8, 5×10^8 p/cm^2). This can be compared from the graph obtained from densitometric scanning of the gel photographs of the samples irradiated at different doses (Figure 6).

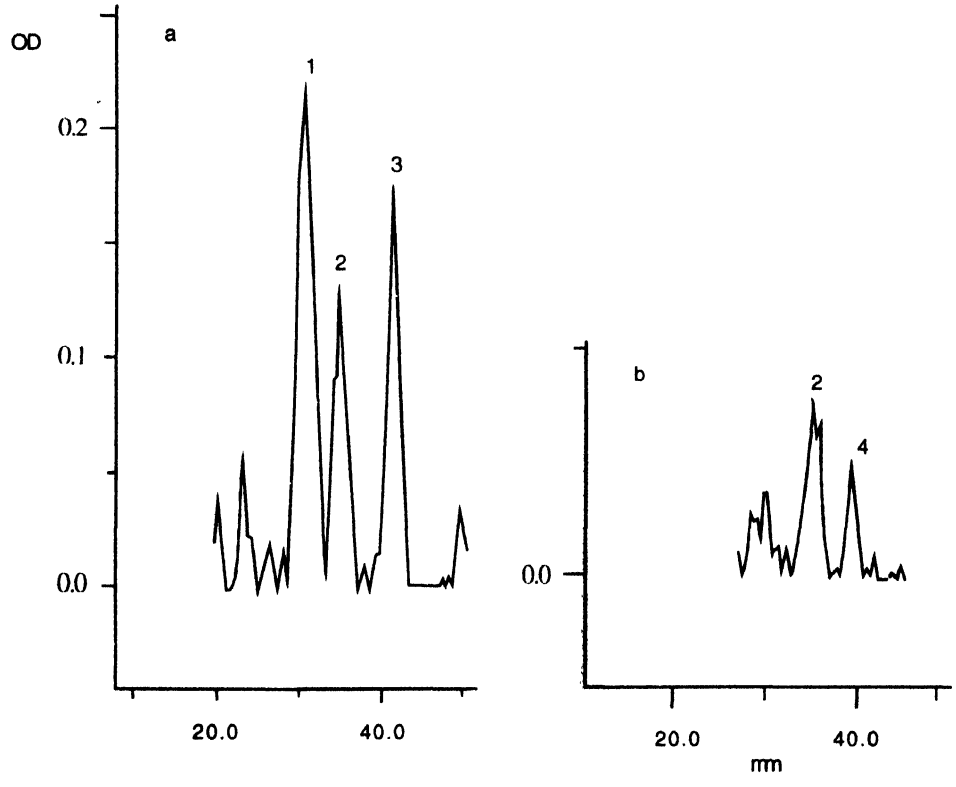

Figure 5. Densitometric tracings obtained by scanning the lanes a and b (marked a and b respectively on the scan) of the gel photographs of Figure 3. Peak 1 indicates Chromosomal DNA, Peak 2 indicates Form II DNA, Peak 3 indicates Supercoiled DNA, Peak 4 indicates Form III (ds break).

Form I_d has been shown to escape bombardment by high energy particles or reactive species at doses that cause Form I DNA significant damage. The chief reason attributed to this, is the more compact nature of the DNA [1,5,15,18]. The partially denatured single stranded structure having duplex region gives this form a unique contour of DNA. The single strand specific probes *e.g.* hydroxyl radical, micrococcal nuclease or S I nuclease *etc.* can access well

this From I_d for digestion while probes requiring double strand, do not. This structure is related to the secondary structure at the replication fork. At the replication fork the partially melted structure maintains a duplex structure which must rewrap nucleosomes after passage of replication machinery through the fork. The advantage of using this Form I_d structure is that,

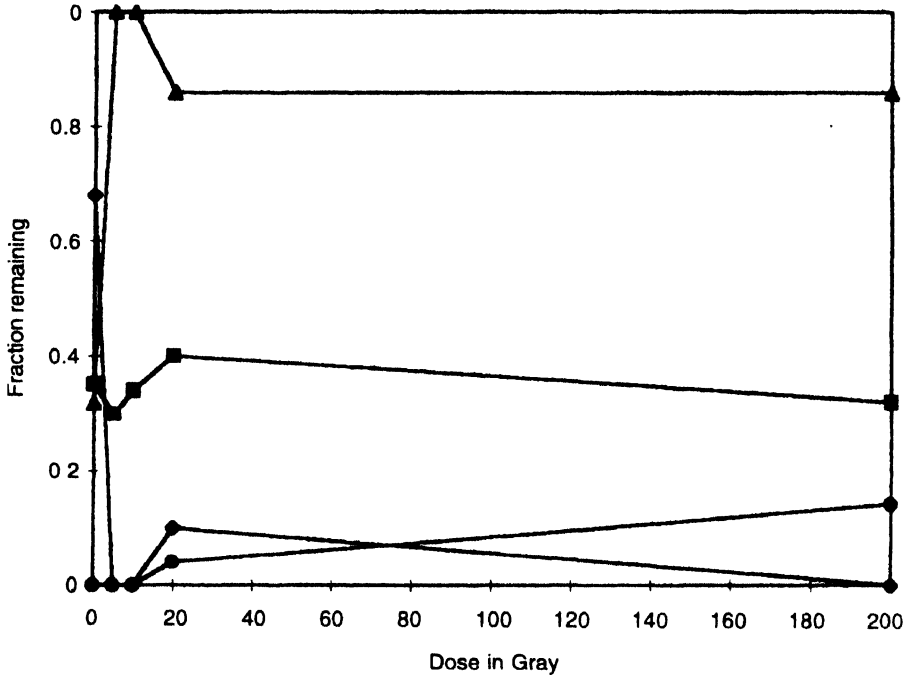

Figure 6. Graphical representation of the densitometric tracings obtained by scanning the negatives of the photograph of samples irradiated at different doses of ^{16}O beam. Solid diamond indicates Form I SC DNA, solid circle indicates ds breakage, solid triangle indicates ss breakage, solid square indicates Form I_d

it is bereft of proteins and yet maintains the nature of the structure. Hence formation of minichromatin on Form I_d is nearest one can achieve in defining repositioning events of nucleosome during eukaryotic replication. The partially unwound structure of Form I_d followed by highly wound structure gives rise its high compactness and hence explains the lesser damage inflicted by radiation or reactive species. Glycerol was used in the samples to prevent the samples from evaporation. Though there was a marked scavenging activity of the glycerol was observed (data not shown) however at such high concentration the ionizing rays could produce SSBs and DSBs on plasmid DNA as also reported by Ayene *et. al.* [19].

The protein bound minichromatin structure are also not susceptible to damage. The protection is attributed to strong binding of the protein to the DNA ; the proteins have provided a shield to the damage of the DNA. The higher doses of irradiation may perhaps escape the protection and can cause DNA strand breakage, but those doses were not tested. Both kinds of Minichromatins were seen to be protected.

The dose profiles in heavy charged particles are such that it is inverse of electromagnetic rays : the deposition of energy increases with depth culminating in a Bragg peak just before the

end of primary particle range. Thus, with proper selection of the dose profiles at a particular depth of the target, the tumour dose can be increased and the dose to the healthy tissue decreased. Thus, using heavy ions with proper selection of dose versus depth would help in

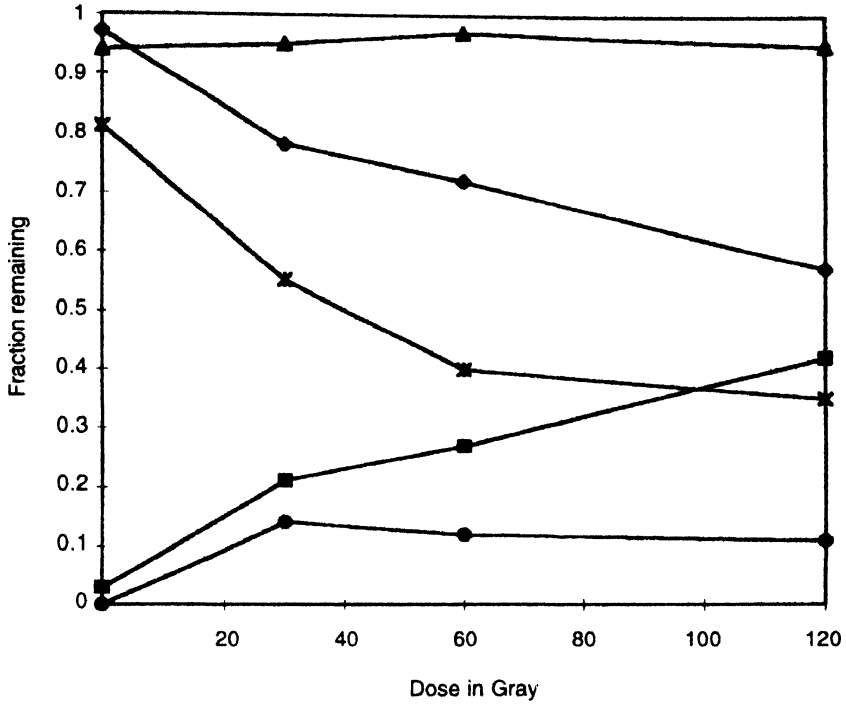

Figure 7. Graph showing nature of damage or protection offered by different samples exposed by γ-rays as derived from Figure 4. Solid diamond indicates Form I SC DNA, solid square indicates single strand break in SC DNA, solid triangle indicates Form I Mitochromatin, solid circle indicates single strand break in Form I_d, X indicates Form I_d mitochromatin.

the clinical trials to determine study of regression of tumour involving fewer side effects. The choice of this strategy of using Bragg peak was followed by earlier workers [20-22] and it is still now used by the present workers for their experiments of radiotherapy [23, 24].

The effect of γ-irradiation :

The γ-irradiation of the samples produces damage to that found in case of ^{16}O beam (Figure. 4). An easy comparison of the nature of damage or protection offered by different samples is drawn in the Figure 7, where the densitometric data obtained from the gel photograph of the different samples exposed to varying doses of γ-rays have been plotted. The mechanism of damage is perhaps slightly different to that of heavy ion damage. The damage observed on Form I_d supercoiled DNA with increasing doses shows single and double strand breakage. The apparent protection of Form I_d is attributed to the compactness due to presence of highly supertwisted secondary structures. These stretches are supposed to make compact structure leading to faster mobility of the DNA in agarose gel.

The histone bound minichromatins on Form I and Form I_d showed protection from the damage. However, the minichromatins on Form I were observed to be better protected than the Form I_d minichromatin. The protection can be attributed either to the altering of the structure of the DNA itself or through scavenging of the radicals [25].

The γ-irradiation causes mutation and cytotoxic damages effecting mainly single and double strand breaks, modifications and damage of the bases and formation of apurinic and apyrimidinic bases. Two mechanisms are suggested for the protection of the DNA from damage *in vitro*. One is due to the compaction of the DNA secondary structure itself and other is due to the secondary structure obtained by binding of proteins or protection offered by protein itself [26].

A comparison of the dose-effect parameter of the heavy ion and γ-ray shows that doses of heavy ions are more effective in damaging the supercoiled DNA than the γ-ray. The samples irradiated with ^{16}O beam at 5×10^6 particle fluence (8 Gray) in presence of 50% glycerol, which is a potent free radical scavenger showed single strand breaks caused at higher doses of γ-ray *i.e.* 60 and 120 Gy. At corresponding doses of the heavy ion, not only single strand breakage was generated but also double strand break started appearing.

4. Conclusion

From the above studies it is clear that the reactive probes which were used, have affected both the Form I or Form I_d DNA in some cases. The Form I_d DNA is an important structure controlling the events of replication initiation. Here we report that the former structure is relatively inert in presence of the ionizing radiations while it is very much susceptible to damage in presence of Hydroxyl radical. We have to address the question why the same species obtained by ionizing rays could not access the Form I_d DNA while it did Form I. Energy deposited during the condensed phases is not distributed uniformly on the microscopic scale. It is deposited in 'spurs' or 50 A in diameter [27]. The concentration of hydrated electrons, hydroxyl radicals and hydrogen atoms in the outer periphery of the spur is not so high, however it is high in the core. Here two parameters are to be considered : one is time magnitude and the other is Z values. The chemical effect of radiation arises at 10^{-12} -- 10^{-7} sec. At this stage C-C and C-N bond breakage occurs along with organic radicals (secondary radicals) begin to act with O_2. Spurs start diffusing to a diameter of 10^4 A. The high stopping power at this region is attributed to the presence of numerous reactive electrons. But this high energy species are distributed in different ways over a total microvolume of 1 μm in diameter [6] which is too diffuse to affect the Form I_d's compactness. But the hydroxyl radicals produced by Fenton's Reaction is retained locally which could access the single stranded regions.

Hence our results show that the proteins acted like a shield to the DNA. The individual amino acids are susceptible to damage to ionizing rays as well as reactive species. Though the C-N and C-C bonds are more prone to damage, the highly compact DNA wrapped around octameric histones in nucleosomes of minichromatins are not affected apparently. This phenomenon is at par with the result obtained by other groups [11, 12, 28].

It is expected that this study would lead to a better understanding of the use of heavy ions in the medical therapy. The high rate of replication, transcription and translation in the rapidly dividing cells of either cancerous growth or developing tissues are particularly

susceptible to damage from radiation. Thus protection offered by the proteins as well as of denatured supercoiled DNA from ionizing radiation should help in determining the dose in terms of actual cell damage. The use of heavy charged particles in radiotherapy potentially represents an advance towards better tumour control and a decrease in morbidity related to radiation, injury of healthy tissues surrounding the target volume. The dose distribution from the oncologist's point view is still difficult to figure out ; however our present study would throw some light on influence of linear energy transfer on the biological effects of its variations related to depth, particle, target and positions of Bragg peak.

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References

[1] J Vinograd, J Lebowitz and R Watson *J Mol Biol* **33** 173 (1968)

[2] J C Wang *J Mol Biol* **89** 783 (1974)

[3] D A Ostrander and H B Gray Jr *Biopolymers* **12** 1387 (1973)

[4] C R Santra and A R Thakur *Int J Biol Macromol* **6** 152 (1984)

[5] C R Santra, S K Mukherjee and A R Thakur *Indian J Biochem Biophys* **30** 123 (1993)

[6] G Kraft, M Kramer and M Scholz *Preprint of Radiation Environment Biophysics* (1992)

[7] G Kraft, W Becher, K Blasche, D Bohne, B Fischer, G Gademann, H Geisel, Th Haberrer, J Klabunde, W Kraft-Weyrather, B Langenbeck, G Munzenberg, S Ritter, W Rosch, D Schardt, H Stetze and Th Schwab *Preprint of Nuclear Tracks and Radiation Research* [1991]

[8] J R Milligan, C C L Wu, J Y-Y Ng, J A Aguilera and J F Ward *Radiat Res* **146** 510 (1996)

[9] E C Friedberg, G C Walker and W Siede DNA *Repair and Mutagenesis* (Washington D C : ASM Press) (1995)

[10] M J Daly and K W Minton *J Bacteriol* **178** 4461 (1996)

[11] J R Milligan, J A Aguilera and J F Ward *Radiat Res* **133** 158 (1993)

[12] H Schuessler, L Distel, R Sieber *Int J Radiat Biol.* **71(5)** 543 (1997)

[13] T A Baker, K Seimizu, B E Funnel and A Kornberg *Cell* **45** 53 (1986)

[14] N L Oleinick, U Balasubramaniam, L Xue and S Chiu *Int. J. Radiat Biol* **66** 523 (1994)

[15] C R Santra, S K Mukherjee and A R Thakur *Indian J Biochem Biophys* **30** 252 (1993)

[16] D J Clark and G Felsenfeld *E M BOJ* **10** 387 (1991)

[17] J W T Spinks and R J Woods *Introduction to Radiation Chemistry* 3E (New York . John Wiley and Sons) (1990)

[18] W Strider, M N Camien and R C Warner *J Biol. Chem.* **256** 7820 (1981)

[19] I S Ayene, C J Koch and R E Krisch *Radiat Res* **146** 501 (1996)

[20] M R Raju, E Bain, S G Carpenter, R A Cox and J B Robertson, *Brit J Radiol.* **51(609)** 704 (1978)

[21] M R Raju, H I Amols, E Bain, S G Carpenter, R A Cox and J B Robertson *Brit J. Radiol* **51(609)** 712 (1978)

[22] M R Raju and S G Carpenter *Brit. J. Radiol.* **51(609)** 720 (1978)

[23] T Kanai, Y Furusawa, K Fukutsu, H Itsukaichi, K Eguchi-Kasai and H Ohara *Irradiation of mixed beam and design of spread-out Bragg Peak for heavy-ion radiotherapy, Radiat. Res* **147(1)** 78-85

[24] D Becker, Y Razskazovskii, M U Callaghan, M D Sevilla *Radiat. Res.* **146** 361 (1996)

[25] K T Wheeler and J V Wieroski *Radiat. Res* **93** 312 (1983)

[26] F Boubrik and J Rouviere-Yaniv *Proc. Natl. Acad. Sci.* (USA) **92** 3958 (1995)

[27] A Singh and H Singh *Prog. Biophys. Molec. Biol.* **39** 69 (1982)

[28] G D D Jones, J R Milligan, J F Ward, P M Calabro-Jones and J A Aguilera *Radiat. Res* **136** 190 (1993)